

1-Deoxy-D-xylulose 5-phosphate reductoisomerase and plastid isoprenoid biosynthesis during tomato fruit ripening

Manuel Rodríguez-Concepción*, Iván Ahumada, Ester Diez-Juez, Susanna Sauret-Güeto, Luisa María Loist, Francesca Gallego, Lorenzo Carretero-Paulet, Narciso Campos and Albert Boronat

Departament de Bioquímica i Biologia Molecular, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1-7, 08028 Barcelona, Spain

*For correspondence (fax: +34 93 402 1219; email: mrodrigu@sun.bq.ub.es)

[†]Present address: Laboratory of Plant Molecular Biology, Rockefeller University, New York, NY 10021-6399, USA.

Summary

The recently discovered 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for the biosynthesis of plastid isoprenoids (including carotenoids) is not fully elucidated yet despite its central importance for plant life. It is known, however, that the first reaction completely specific to the pathway is the conversion of 1-deoxy-D-xylulose 5-phosphate (DXP) into MEP by the enzyme DXP reductoisomerase (DXR). We have identified a tomato cDNA encoding a protein with homology to DXR and *in vivo* activity, and show that the levels of the corresponding *DXR* mRNA and encoded protein in fruit tissues are similar before and during the massive accumulation of carotenoids characteristic of fruit ripening. The results are consistent with a non-limiting role of DXR, and support previous work proposing DXP synthase (DXS) as the first regulatory enzyme for plastid isoprenoid biosynthesis in tomato fruit. Inhibition of DXR activity by fosmidomycin showed that plastid isoprenoid biosynthesis is required for tomato fruit carotenogenesis but not for other ripening processes. In addition, dormancy was reduced in seeds from fosmidomycin-treated fruit but not in seeds from the tomato *yellow ripe* mutant (defective in phytoene synthase-1, *PSY1*), suggesting that the isoform *PSY2* might channel the production of carotenoids for abscisic acid biosynthesis. Furthermore, the complete arrest of tomato seedling development using fosmidomycin confirms a key role of the MEP pathway in plant development.

Keywords: isoprenoid, carotenoid, fosmidomycin, reductoisomerase, ripening, dormancy.

Introduction

Plants synthesize tens of thousands of isoprenoids (also known as terpenoids). These compounds constitute one of the most diverse groups of natural products and have distinct roles in membrane structure, redox chemistry, growth regulation, signalling, and defence mechanisms. Despite their diversity, all isoprenoids derive from a common building unit, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). It is now well established that there are two pathways for IPP biosynthesis in plants (reviewed by Lichtenthaler *et al.*, 1997; Lichtenthaler, 1999). Cytoplasmic and mitochondrial isoprenoids are formed from acetyl CoA via mevalonate, whereas plastidial isoprenoids (including monoterpenes, gibberellins, abscisic acid, carotenoids, and the prenyl moiety of chlorophylls, plastoquinone, and tocopherol) derive from a mevalonate-independent pathway currently

known as the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (Figure 1). The MEP pathway was first discovered in bacteria by Rohmer *et al.* (1993), and is best characterized in *Escherichia coli*. The initial reaction involves the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) from pyruvate and D-glyceraldehyde 3-phosphate, and is catalysed by DXP synthase (DXS) (Lange *et al.*, 1998; Lois *et al.*, 1998). In the second step, DXP is converted to MEP by the enzyme DXP reductoisomerase (DXR) (Lange and Croteau, 1999; Schwender *et al.*, 1999; Takahashi *et al.*, 1998). Although other genes involved in the steps from MEP to IPP have been described in *E. coli* (Campos *et al.*, 2001; Herz *et al.*, 2000; Lüttgen *et al.*, 2000; Rohdich *et al.*, 1999), the complete pathway remains to be elucidated.

After identification of the *E. coli* gene encoding DXS, it was found that a homologous gene was disrupted in the

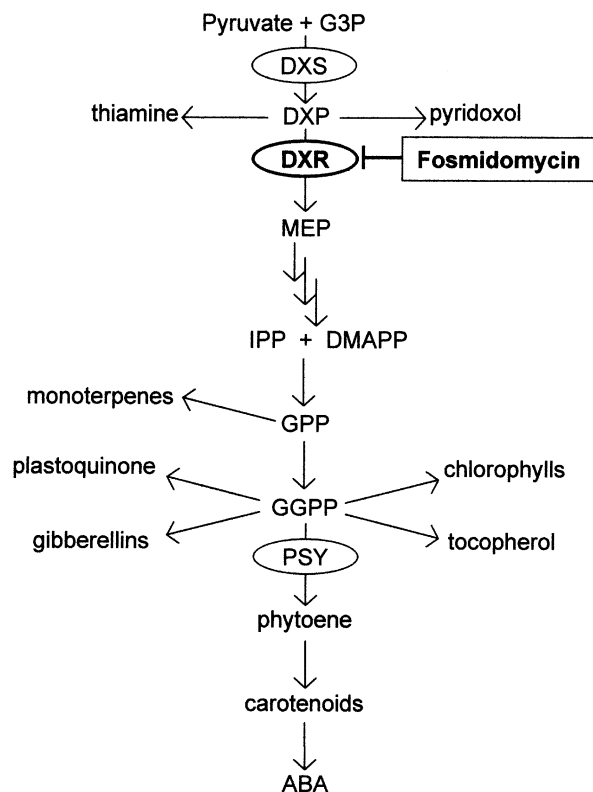


Figure 1. Plastid isoprenoid biosynthesis pathway. G3P, D-glyceraldehyde 3-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MEP, 2-C-methyl-D-erythritol 4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; ABA, abscisic acid.

previously reported *cla1* mutant of *Arabidopsis thaliana* (Lois *et al.*, 1998; Mandel *et al.*, 1996). Mutant *cla1* plants show albinism and a very early arrest of chloroplast development (Estévez *et al.*, 2000; Mandel *et al.*, 1996). Despite the null mutation of the *CLA1* gene, chlorophyll and carotenoids are still detectable in the mutant plants (Estévez *et al.*, 2000), which may be explained by the presence of a second gene encoding DXS in *Arabidopsis* (Araki *et al.*, 2000). As DXP is an intermediate not only in IPP biosynthesis but also in thiamine and pyridoxol biosynthesis in plastids (Figure 1; Julliard and Douce, 1991; Julliard, 1992), it is possible that at least some features of the *cla1* phenotype are not due exclusively to the absence of isoprenoid products but also to the block in the biosynthesis of thiamine and pyridoxol. The conversion of DXP to MEP catalysed by DXR is actually the first step specific for isoprenoid biosynthesis in plastids (Figure 1). Therefore, a block of DXR activity should more clearly show the specific effects of inhibited plastid isoprenoid biosynthesis on plant development. Although plant mutants lacking DXR activity have not been identified

yet, a DXR-null phenotype can be obtained by inhibiting enzyme activity using fosmidomycin, a transition-state analogue in the conversion of DXP to MEP by DXR. Fosmidomycin has been shown to inhibit DXR activity in plants (Mueller *et al.*, 2000; Schwender *et al.*, 1999), leading to a specific block in the synthesis of plastid isoprenoids (Sharkey *et al.*, 2001; Zeidler *et al.*, 1998).

Fruit development in tomato (*Lycopersicon esculentum*) constitutes a good system to investigate the regulation of plastid isoprenoid biosynthesis. The main synthesis of isoprenoids takes place during ripening, a developmental process that involves a dramatic accumulation of carotenoids (particularly lycopene) and degradation of chlorophylls, causing the fruit colour to change from green to red (Bartley and Scolnik, 1995; Gillaspay *et al.*, 1993; Khudairi, 1972). Tomato proteins homologous to the *E. coli* enzymes involved in isoprenoid biosynthesis have recently been identified (Lois *et al.*, 2000; Rohdich *et al.*, 2000). However, the contribution of each enzyme to the biosynthesis of final plastid isoprenoid products remains to be established. DXS and DXR both appear to be potential candidates for control of the flux of plastid isoprenoid precursors. Using tomato fruit development as a model system, we proposed a regulatory role for DXS in carotenoid biosynthesis (Lois *et al.*, 2000). In order to characterize the role of DXR, we have studied the pattern of mRNA and protein accumulation during tomato fruit ripening. Our results support a non-limiting role of DXR for plastid isoprenoid biosynthesis in tomato fruit. The block of DXR activity with fosmidomycin, however, resulted in inhibited carotenoid synthesis and reduced seed dormancy, but did not affect other aspects of ripening.

Results

Identification of a tomato cDNA clone encoding DXR

To identify tomato sequences encoding DXR, we first searched the databases available on the National Center for Biotechnology Information (NCBI) web page. Using the amino acid sequence from peppermint DXR (Lange and Croteau, 1999) as a query, we identified several tomato EST clones showing homology to different regions of the protein. Complete sequencing of clone AI483173 (cLEB8M11) showed that it contains a cDNA of 1727 bp and an ORF of 1425 bp encoding a protein of 475 amino acid residues and predicted molecular mass of 51.5 kDa. As shown in Figure 2(a), the predicted tomato protein is highly similar to DXR proteins from *Catharantus roseus* (87.3% identity), *Zea mays* (86.5%), *Arabidopsis thaliana* (85.7%) and *Mentha x piperita* (81.4%). The N-terminal domain of these proteins, however, is poorly conserved (Figure 2a). This N-terminal region, which is not present in

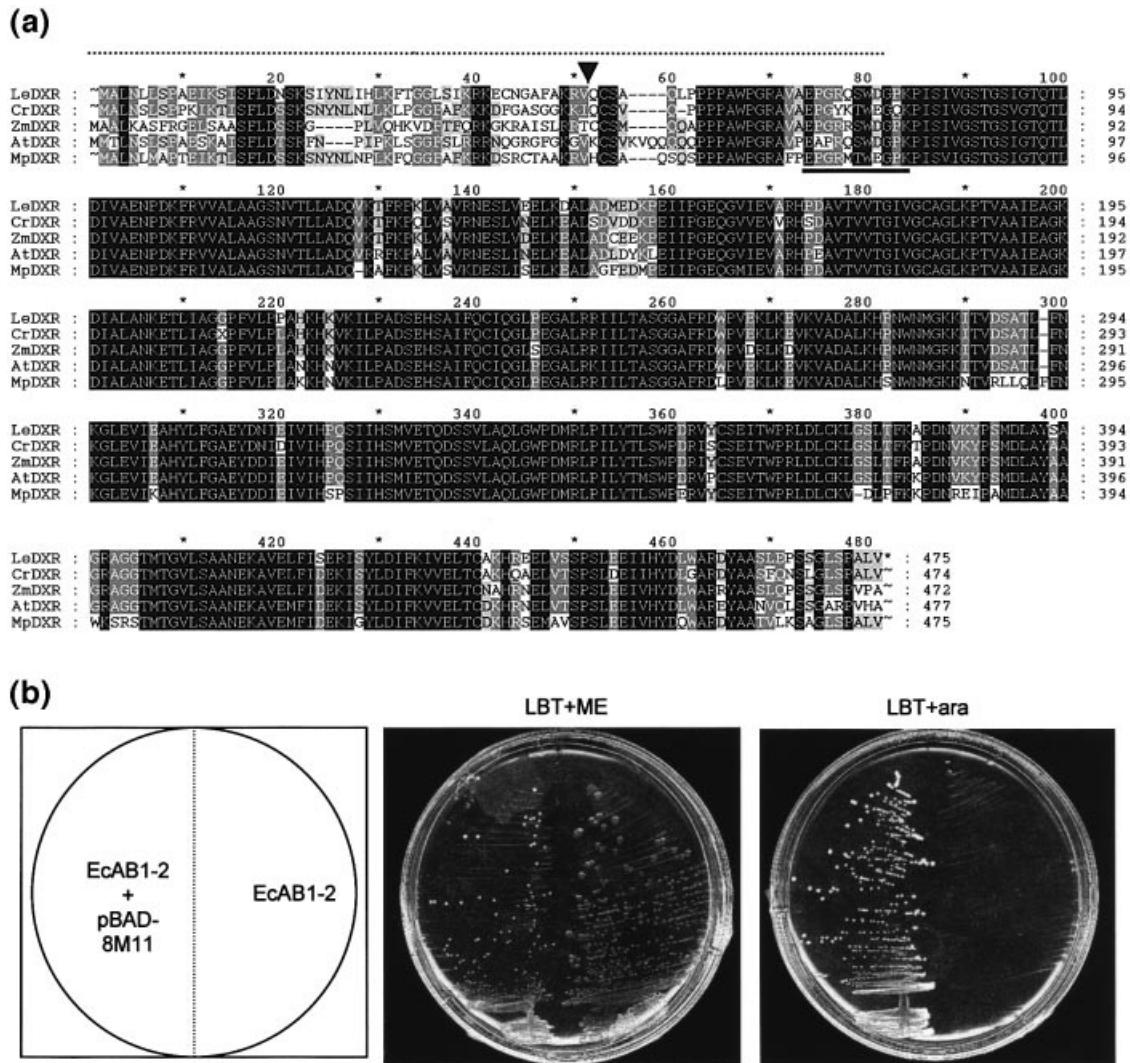


Figure 2. Identification of tomato DXR.

(a) Multiple alignment of the protein sequence deduced from the tomato (*Lycopersicon esculentum*) cLEB8M11 clone with DXR sequences from *Catharantus roseus* (accession number AAF65154.1), *Zea mays* (CAC03581.1), *Arabidopsis thaliana* (AAF73140.1) and *Mentha x piperita* (AAD24768.1). Identical residues are shown in white inside black boxes (when they are conserved in the five sequences), in white inside grey boxes (conserved in four sequences), and in black inside grey boxes (conserved in three sequences). The *Arabidopsis* peptide used for antibody production is underlined. The N-terminal domain absent in bacterial DXR enzymes is indicated with a dotted line. A predicted cleavage site of the plastid targeting peptide is indicated with an arrowhead.

(b) Complementation assay of the *E. coli* *dxr* mutant. *E. coli* MC4100*dxr::TET* cells (strain EcAB1-2) were transformed with plasmid pBAD-8M11 (in which the expression of the protein encoded by the tomato cLEB8M11 clone is under the control of the arabinose-inducible P_{BAD} promoter) and grown on LB plates supplemented with tetracycline and either 0.5 mM ME or 0.02% arabinose (left side of the plates). Untransformed EcAB1-2 strain was grown on the same plates as a control (right side).

the microbial DXR enzymes, functions as a plastid targeting sequence in *Arabidopsis* (LCP, NC and AB, unpublished results), consistent with the plastidial localization of the MEP pathway. Genomic DNA blot hybridization experiments using the isolated cDNA as a probe showed a pattern of bands consistent with the presence of a single gene encoding DXR in tomato (data not shown).

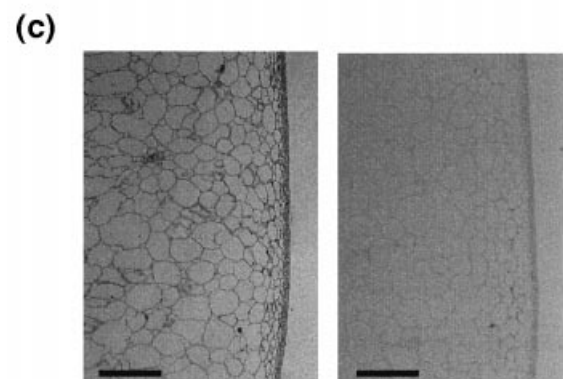
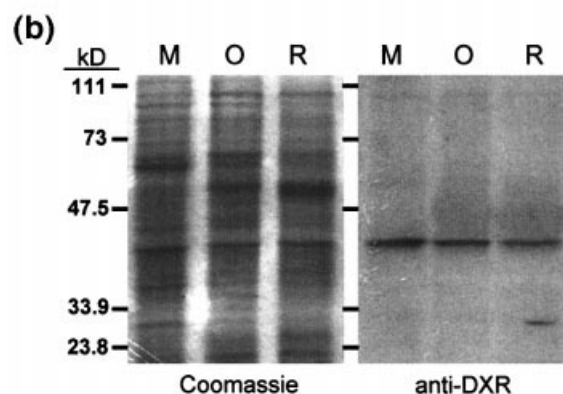
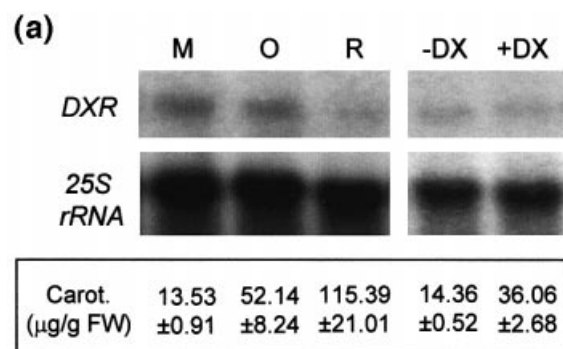
To confirm the biochemical function of the protein encoded by the cDNA from clone cLEB8M11, a comple-

mentation assay was carried out using the *E. coli* strain EcAB1-2 (Rodríguez-Concepción *et al.*, 2000), in which the chromosomal *dxr* gene was disrupted by insertion of the *TET* marker conferring tetracycline resistance. Although this disruption is lethal, mutant EcAB1-2 cells can grow in media supplemented with 2-C-methyl-D-erythritol (ME) (Rodríguez-Concepción *et al.*, 2000). For the complementation assay, plasmid pBAD-8M11 was created by subcloning the entire coding sequence from clone cLEB8M11 into

the pBAD expression vector under the control of the P_{BAD} promoter, which can be activated with arabinose. In EcAB1-2 cells transformed with pBAD-8M11, induction with arabinose overcame ME auxotrophy (Figure 2b). These results demonstrate that the cloned tomato cDNA encoded a functional DXR.

Temporal and spatial distribution of DXR mRNA and encoded protein in tomato fruit

As a first approach to investigate the contribution of DXR to carotenoid biosynthesis in tomato fruit, we analysed the pattern of accumulation of *DXR* transcripts by RNA blot hybridization using the full-length cLEB8M11 cDNA as a probe. Unlike *DXS* (Lois *et al.*, 2000), *DXR* expression was



not induced during the transition from mature green to orange fruit (when the surge in carotenoid biosynthesis takes place) or in further stages of fruit ripening (Figure 3a). Carotenoid accumulation can also be induced by feeding mature green fruit with 1-deoxy-D-xylulose (DX) (Lois *et al.*, 2000). DX treatment, however, did not result in increased levels of *DXR* mRNAs either (Figure 3a). These results suggest that enhanced biosynthesis of carotenoids during fruit ripening may not require an induction of *DXR* expression.

To address the possibility of post-transcriptional control mechanisms that might result in increased protein levels, we immunodetected DXR in tomato fruit extracts using an antibody raised against an 11 amino acid peptide from the N-terminal region of the *Arabidopsis* enzyme (Figure 2a). This antibody, which recognizes the tomato protein expressed in *E. coli* (data not shown), detected a single cross-reacting band of about 45 kDa in fruit protein extracts (Figure 3b). This result is consistent with the presence of a mature DXR protein in which the N-terminal plastidial signal peptide has been processed after import of the protein into plastids (see above, Figure 2a). Figure 3(b) shows that levels of protein recognized by the anti-DXR antibody in tomato fruit at the developmental stages of mature green (at the onset of ripening), orange (at the stage of maximum carotenoid biosynthesis) and red (ripe) are consistent with the *DXR* mRNA accumulation pattern shown in Figure 3(a), confirming that similar DXR protein levels were present throughout tomato fruit development. We have previously shown that *DXS* mRNA was predominantly localized in the outer layers of cells of the orange fruit pericarp, where the main accumulation of carotenoid pigments takes place (Lois *et al.*, 2000). By contrast, *in situ* hybridization experiments on tomato orange fruit sections showed that transcripts encoding

Figure 3. Analysis of *DXR* expression during tomato fruit ripening. (a) RNA blot analysis of *DXR* expression during fruit ripening. Tomato mature green (M), orange (O) and red ripe (R) fruit were harvested directly from the plant. In a different experiment, mature green fruit were longitudinally cut in half and incubated for 3 days after injection of each half with either a DX (+) or a control (-) solution. All the samples were used for both RNA blot analysis with a *DXR* probe and total carotenoid measurement. A 25S rRNA probe was used to compare the RNA amounts loaded in each lane. After hybridization, blots were exposed for 4 and 2 days, respectively. The total carotenoid amount ($\mu\text{g g}^{-1}$ fresh weight) in the same fruit samples used for RNA isolation is indicated (mean and standard errors of three measurements). (b) Immunoblot analysis of DXR accumulation. SDS-PAGE with extracts from tomato mature green (M), orange (O) and red ripe (R) fruit was used for Coomassie staining to monitor loading (left) and immunoblot analysis with a specific anti-DXR antiserum (right). The position and mass (in kDa) of size standards is indicated. (c) Localization of *DXR* transcripts on tomato orange fruit sections by *in situ* hybridization. Sections ($8\mu\text{m}$) from orange fruit pericarp were incubated with tomato *DXR* antisense (left panel) or sense (right panel) riboprobes. Bars = 0.5 mm.

DXR were evenly distributed throughout the pericarp tissue (Figure 3c), confirming that cells accumulating carotenoids do not require higher levels of *DXR* expression. Together, our results are consistent with a non-limiting role of DXR for isoprenoid biosynthesis during tomato fruit ripening.

Inhibition of fruit plastidic isoprenoid biosynthesis with fosmidomycin

The role of DXR and plastid isoprenoids in tomato fruit ripening was studied by blocking DXR activity with fosmidomycin, a strong and specific inhibitor of plant DXR (Mueller *et al.*, 2000; Schwender *et al.*, 1999; Sharkey *et al.*, 2001; Zeidler *et al.*, 1998). Fosmidomycin was injected into fruit just prior to the onset of ripening, at the mature green stage. Experiments were carried out with fruit collected from the plant and cut in half longitudinally. After estimating the volume of each half from its diameter, fosmidomycin was applied to the placenta of one of the halves at a final concentration of 100 μM , and a control solution without the inhibitor was applied to the other half. Following incubation of both halves at 25°C for 5 days, treated and control halves were frozen in liquid nitrogen and ground to a fine powder which was used for analysis of chlorophyll and carotenoid contents and mRNA levels (Figure 4).

Control halves synthesized carotenoids and became red in a fashion similar to intact fruit. In contrast, halves treated with fosmidomycin turned yellow-orange but did not accumulate carotenoids (Figure 4), most likely because of the absence of isoprenoid precursors. Treatment with fosmidomycin, however, significantly induced the accumulation of transcripts from genes involved in carotenoid biosynthesis such as *DXR*, *DXS* and *PSY1* (the gene encoding the isoform of phytoene synthase responsible for carotenoid biosynthesis in ripening fruit; Figure 1) (Bartley and Scolnik, 1995; Fraser *et al.*, 1999). In contrast to carotenoid accumulation, chlorophyll degradation (an event that takes place early during ripening; Fraser *et al.*, 1994; Khudairi, 1972) was not altered by fosmidomycin (Figure 4), indicating that blocking plastid isoprenoid biosynthesis did not affect other processes associated with ripening. To further study the effects of fosmidomycin treatment on fruit ripening besides pigment accumulation, expression of the *ACS2* gene was monitored by RNA blot analysis. Tomato *ACS2* encodes an isoform of 1-aminocyclopropane-1-carboxylate (ACC) synthase, the enzyme that catalyses the first reaction of the ethylene biosynthesis pathway. *ACS2* transcript levels closely parallel ethylene accumulation during tomato fruit development and can therefore be used as a reliable indication of fruit ethylene biosynthesis (Nakatsuka *et al.*, 1998). The level of

ACS2 mRNA in fosmidomycin-treated fruit halves was similar to that in control halves (Figure 4), suggesting that fosmidomycin treatment did not affect ethylene biosynthesis in tomato fruit. In summary, the data suggest that plastid isoprenoid biosynthesis is required for carotenogenesis but not for other developmental processes associated with ripening.

Dormancy is reduced in seeds from fosmidomycin-treated fruit

During fruit ripening, seeds develop and mature, becoming dormant in a process mostly controlled by the carotenoid-derived hormone ABA (Zeevaart and Creelman, 1988). The effect of inhibited DXR activity and plastid isoprenoid biosynthesis on seed dormancy was studied by injecting fosmidomycin to a final concentration of 200 μM directly into mature green fruit attached to the plant. The same volume of a solution without fosmidomycin was injected to control fruit. As described above for fruit halves, fosmidomycin-treated fruit became yellow-orange when ripe (Figure 5a). Apart from the colour, control and fosmidomycin-treated fruit were indistinguishable in terms of ripening time, softness, or any other obvious feature. No significant differences in germination percentages of freshly harvested seeds from ripe control and fosmidomycin-treated fruit were found either. However, seeds from fruit treated with the inhibitor germinated significantly earlier than seeds from control fruit (Table 1). To study whether this reduced dormancy was a consequence of inhibited biosynthesis of carotenoids for ABA production, we used the tomato *yellow flesh* (*r*) mutant. The *r* mutation results in a non-functional *PSY1* enzyme and a block in fruit carotenoid synthesis during ripening (Fray and Grierson, 1993). However, dormancy was not altered in seeds from the *r* mutant compared to wild-type seeds (Table 1). Similar results were observed when seeds were germinated in medium supplemented with 100 μM fosmidomycin (Table 1), suggesting that the reduced dormancy of seeds from fosmidomycin-treated fruit was not due to inhibited plastid isoprenoid biosynthesis during germination but was acquired during seed development. In this medium, seedlings showed an albino phenotype (Figure 5c) consistent with the phenotype of the *Arabidopsis cla1* mutant, in which a genetic block of plastid isoprenoid synthesis leads to albinism (Mandel *et al.*, 1996).

Development of seedlings from seeds of fosmidomycin-treated and control fruit was very similar (Figure 5b,c). In the presence of fosmidomycin, however, growth was limited and only a few albino leaflets developed before seedlings died (Figure 5d). In summary, our data suggest that inhibition of plastid isoprenoid production during fruit ripening and seed development results in reduced seed

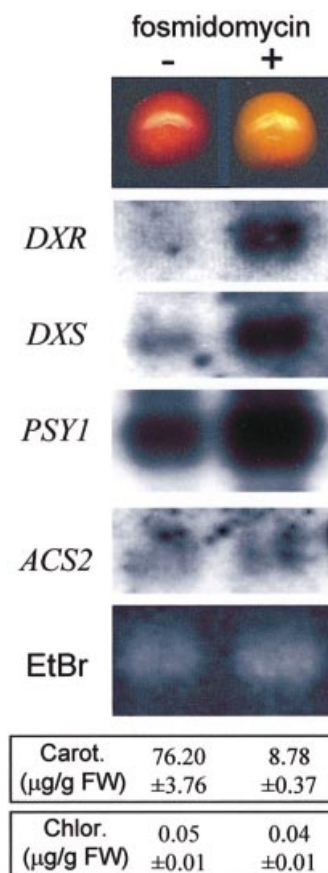


Figure 4. Effect of fosmidomycin injection on tomato fruit gene expression and pigment content.

Fruit were cut in half longitudinally. After estimating the volume of each half from its diameter, fosmidomycin was applied to one of the halves to a final concentration of 100 μM (+), and a control solution without the inhibitor was applied to the other half (-). After 5 days, treated and control halves were frozen in liquid nitrogen and ground to a fine powder which was used for both RNA blot analysis and measurement of carotenoid and chlorophyll contents. Blots were hybridized with probes for *DXR*, *DXS*, *PSY1* and *ACS2*, as indicated. Ethidium bromide staining of the 25S rRNA is included to show that similar amounts of RNA were loaded. Total carotenoid and chlorophyll amounts ($\mu\text{g g}^{-1}$ fresh weight) in the same fruit samples used for RNA isolation are indicated (mean and standard errors of three measurements).

Table 1 Germination of freshly harvested seeds from control and fosmidomycin-treated fruit

Fruit (treatment)	MS	MS + 100 μM fosmidomycin
Wild-type (fosmidomycin)	2.63 $\pm$ 0.80	2.71 $\pm$ 0.75
Wild-type (control)	5.75 $\pm$ 1.97	6.33 $\pm$ 1.86
Yellow ripe, <i>r</i> (untreated)	5.32 $\pm$ 1.17	4.96 $\pm$ 0.85

Seeds were germinated on solid MS medium with or without 100 μM fosmidomycin. Following imbibition at 4°C for 5 days, seeds were incubated in a growth chamber at 25°C. Numbers indicate day of germination after imbibition (mean $\pm$ SD).

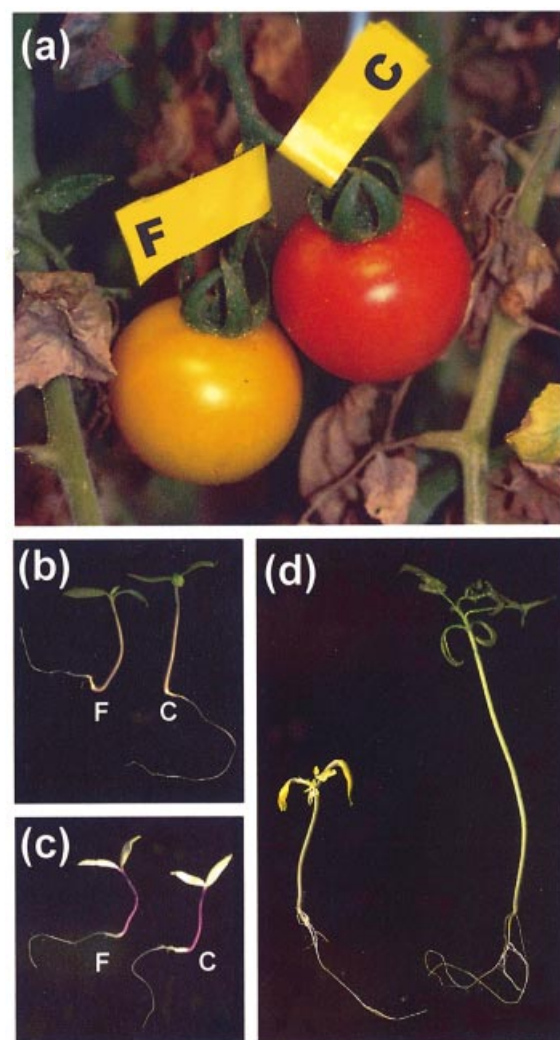


Figure 5. Fosmidomycin effect on tomato plant development.

(a) After estimating the volume of a fruit attached to the plant from its diameter, fosmidomycin was injected to a final concentration of 200 μM (F). A solution without the inhibitor was applied to control fruit of the same inflorescence (C). The image shows fruit 2 weeks after treatment. (b) Six-day-old seedlings from seeds collected from fruit shown in (a) germinated on MS medium. (c) Six-day-old seedlings from seeds collected from fruit shown in (a) germinated on MS medium supplemented with 100 μM fosmidomycin. (d) Twenty-day-old seedlings from seeds collected from control fruit grown on MS medium either without (right) or with 100 μM fosmidomycin (left).

dormancy. They also show that *DXR* activity and plastid isoprenoid biosynthesis are required for tomato plant development.

Discussion

The recently discovered (and yet not fully elucidated) MEP pathway for the biosynthesis of plastid isoprenoids represents a central metabolic pathway for plant life. In spite of

its importance from both basic and applied perspectives, the mechanisms of regulation of the pathway have remained largely unknown. Since the identification of DXS and DXR, the enzymes catalysing the first two steps of the MEP pathway, both have been tentatively proposed as potential control points. Using tomato fruit as a model system, we have previously shown the reaction catalysed by DXS to be a key regulatory step in carotenoid biosynthesis (Lois *et al.*, 2000). A regulatory role of DXS has also been proposed in bacteria (Harker and Bramley, 1999; Miller *et al.*, 2000). DXS, however, not only synthesizes DXP as a precursor for plastid isoprenoid biosynthesis, but also for the production of thiamine and pyridoxol (Julliard and Douce, 1991; Julliard, 1992). It was therefore proposed that the next step of the pathway, the committed biosynthesis of MEP by DXR, could represent another rate-limiting point for the specific biosynthesis of isoprenoids in plastids (Lange and Croteau, 1999; Takahashi *et al.*, 1998). The data reported here, however, do not support such a limiting role for DXR. Thus, the massive carotenoid accumulation that takes place during fruit ripening does not seem to require increased levels of DXR transcripts and encoded protein (Figure 3). As ripening is a developmental process unique to fruit, it is possible that the non-limiting nature proposed for DXR is fruit-specific. However, whereas over-expression of *Synechococcus* DXR in *E. coli* did not lead to increased DMAPP levels, DXS over-expression did, suggesting that DXS but not DXR catalyses a rate-limiting step in bacteria also (Miller *et al.*, 2000). On the other hand, a strong induction of both DXS and DXR transcript accumulation was observed in several monocot species following colonization of roots by mycorrhizal fungi, in correlation with concomitant accumulation of specific apocarotenoids (Walter *et al.*, 2000). Together, it is possible that co-ordinated regulation of at least the first two enzymes of the pathway was important for the production of specific plastid isoprenoids under certain circumstances in at least some plants. Transgenic plants expressing DXS and DXR should provide further evidence to determine the role of each enzyme in each developmental stage.

Fosmidomycin treatment resulted in a block in fruit carotenoid accumulation during ripening, despite induction of the expression of genes involved in carotenogenesis, such as DXS, DXR and PSY1 (Figure 4). It is likely that an eventual increase in DXS and DXR levels was not sufficient to rescue inhibition of DXR activity by fosmidomycin, resulting in the absence of plastid IPP and GGPP that could be channelled to carotenoid biosynthesis by PSY activity. The block of DXR activity with fosmidomycin might cause an accumulation of DXP (Figure 1), which might in turn be responsible for activation of DXS and PSY1 expression, as suggested from experiments in which DX was directly injected into fruit (Lois *et al.*, 2000). This

mechanism, however, would not explain the increase in DXR transcript level in fosmidomycin-treated fruit halves, since DXR expression appears not to be induced by DX (Figure 3a). Alternatively, low levels of an isoprenoid precursor downstream of MEP may result in an up-regulation of the expression of DXR (and, perhaps, also DXS and/or PSY1). The up-regulation of DXR in response to inhibited plastid isoprenoid biosynthesis in fruit is compatible with a non-limiting role of the enzyme. It is possible that when plastid isoprenoid biosynthesis drops below the threshold required for normal cell functions, a feedback mechanism might exist to restore DXR expression back to 'house-keeping' levels.

Although fosmidomycin inhibited carotenoid accumulation, fruit showed a yellow-orange colour when ripe (Figures 4 and 5). A similar phenotype is observed in ripe fruit from the tomato *r* mutant, in which the presence of a non-functional PSY1 enzyme prevents carotenoid accumulation during ripening (Fray and Grierson, 1993). It is likely that the yellow-orange colour of fosmidomycin-treated fruit results from accumulation of the flavonoid chalconaringenin, as reported for the mutant *r* fruit (Fray and Grierson, 1993). Unlike the *r* mutation, however, fosmidomycin treatment depletes fruit not only of carotenoids (the main isoprenoid product synthesized during ripening) but also of the remaining plastid isoprenoids (Zeidler *et al.*, 1998). According to our data (Figure 4), those other isoprenoid products do not appear to influence other aspects of ripening such as softening, chlorophyll degradation and ethylene biosynthesis (as estimated from ACS2 expression; Nakatsuka *et al.*, 1998). By contrast, some specific plastid isoprenoids might have a role in seed dormancy. The observation that seeds from fosmidomycin-treated fruit germinated earlier than seeds from control fruit whereas dormancy of mutant *r* seeds was not altered compared to wild-type seeds (Table 1) suggests that a plastid isoprenoid product that is present in both wild-type and mutant *r* fruit has a role in inducing seed dormancy. The reduced seed dormancy phenotype described in fosmidomycin-treated fruit might be due to reduced levels of ABA, a carotenoid-derived plant hormone (Cutler and Krochko, 1999; Zeevaart and Creelman, 1988). It is well known that treatment of tomato fruit with inhibitors of carotenoid and ABA biosynthesis results in reduced seed dormancy (Zeevaart and Creelman, 1988), and the tomato ABA-deficient *sitiens* mutant produces seeds that readily germinate compared to wild-type seeds (Groot and Karssen, 1992). In addition, it cannot be excluded that the ABA required for seed dormancy is present in mutant *r* fruit. Apart from the PSY1 isoform that is responsible for carotenoid accumulation in ripening fruit, tomato plants have a second isoform (PSY2) mainly involved in the production of young fruit and leaf carotenoids (Bartley and Scolnik, 1993; Fraser *et al.*,

1999). PSY2 is present in ripening tomato fruit as well, where it has been proposed to channel the carotenoid pathway to the production of end-products different from those produced through PSY1 activity (Fraser *et al.*, 1999). In fact, in ripening fruit from the *r* mutant (with a mutant *PSY1* but an intact *PSY2* gene), the amount of carotenes was much more reduced (0.6% of the wild-type level) than the amount of xanthophylls (12%). As ABA is synthesized from xanthophylls (Cutler and Krochko, 1999), it may be speculated that PSY2 might channel plastid GGPP to the production of xanthophylls for ABA synthesis, therefore preventing alterations of dormancy in seeds from the *r* mutant. It would be interesting to study the localization of PSY2 in fruit and seed tissues, as it is known that tomato seed dormancy does not depend on actual ABA levels in mature seeds or fruit but is regulated by the ABA fraction in embryo and endosperm tissues during seed development (Groot and Karssen, 1992).

Finally, we observed that seedling development is heavily impaired in the presence of fosmidomycin (Figure 5), suggesting that plastid isoprenoids have a key role in plant developmental processes. The albino phenotype of the tomato seedlings growing in the presence of fosmidomycin is reminiscent of that observed in *Arabidopsis cla1* seedlings (Mandel *et al.*, 1996), suggesting that albinism is caused by a block in plastid isoprenoid biosynthesis rather than a block in the production of thiamine and pyridoxol. Detailed analysis of plants grown in the presence of fosmidomycin should determine whether the specific developmental processes altered in the *Arabidopsis cla1* mutant such as chloroplast development and mesophyll tissue formation (Estévez *et al.*, 2000; Mandel *et al.*, 1996) are caused by the specific block in plastid isoprenoid synthesis. Identification of DXR mutants and construction of transgenic plants with altered expression of *DXR* and other genes of the MEP pathway will provide a deeper insight into the role of this pathway in specific aspects of plant development.

Experimental procedures

Plant material and treatments

Growth conditions of tomato (*Lycopersicon esculentum* Mill. cv Ailsa Craig) wild-type and mutant *yellow flesh (r)* plants and fruit development stages were as previously described (Lois *et al.*, 2000). A stock solution of 0.1 M fosmidomycin (Gateway Chemical Technology) was prepared in 10 mM Tris-HCl pH 8.5. For experiments with fruit halves, mature green fruit was removed from the plant and cut in half longitudinally. Immediately, the required volume of stock solution to give a final concentration of 100 μ M fosmidomycin was applied with a Gilson pipetter to the placenta of one of the halves after estimating the volume of the half from its diameter. The equivalent volume of 10 mM Tris-HCl pH 8.5 was applied to the other half as a control. Afterwards, both halves were protected from desiccation with plastic wrap, incubated at

25°C for 5 days, and then photographed, frozen in liquid nitrogen, and stored at -80°C until used. Injection of the fosmidomycin stock solution or the control solution without the inhibitor into mature green fruit attached to the plant was carried out with a 705-N Hamilton syringe (Sigma). Selected fruit were labelled and their volume was estimated from their diameter. After injection, untreated fruit from the same inflorescence were removed. Treated fruit were left attached to the plant. Seeds were extracted from over-ripe fruit, washed with tap water to remove the remnants of fruit tissue, and dried at room temperature. For germination, duplicates of 16–25 seeds were sown in Magenta boxes on MS medium with or without 100 μ M fosmidomycin. After imbibition and vernalization for 5 days at 4°C in the dark, seeds were germinated at 25°C under long-day conditions (16 h light/8 h dark).

CDNA identification and sequence analysis

Clone cLEB8M11 (GeneBank accession number AI483173) was identified after a BLAST search on the NCBI web page (<http://www.ncbi.nlm.nih.gov>) using the protein sequence from pepper-mint DXR (Lange and Croteau, 1999) as a query. The plasmid clone was obtained from David Frisch (Clemson University Genomics Institute, Clemson, GA, USA), and both strands of the cloned cDNA were sequenced with the ABI PRISM DNA sequencing kit (Amersham) using vector primers and specific primers. Sequence analyses were performed using the GCG 9.0 software package (Genetics Computer Group Inc.) and the NCBI web programs. Multiple alignment was performed using the PILEUP program (GCG) with the default options. Plastid targeting peptides were predicted with ChloroP (genome.cbs.dtu.dk/services/chlorop).

E.coli complementation

The entire coding sequence from the cDNA in plasmid clone cLEB8M11 was amplified using Pfu DNA polymerase (Promega) and primers LeDXR5F (5'-GAATTCGCACGAGCCCATC-3') and LeDXR3R (5'-AAAGGGTGATGATGTAGTAC-3'). The PCR product was then cloned into plasmid pBAD-GFPuv (Clontech) after removing the GFPuv sequence by digestion with *NheI* and *EcoRI*. The resulting plasmid pBAD-8M11 was used to transform *E. coli* EcAB1-2 cells, in which the *dxr* gene in strain MC4100 was disrupted by insertion of the *TET* marker conferring tetracycline resistance (Rodríguez-Concepción *et al.*, 2000). Positive transformants complementing the *dxr* mutation were selected on LB plates supplemented with 7.5 μ g ml⁻¹ tetracycline, 100 μ g ml⁻¹ ampicillin and 0.02% arabinose to induce expression from the *P_{BAD}* promoter. When required, plates were supplemented with ME as described (Rodríguez-Concepción *et al.*, 2000).

RNA blot analysis

For fruit RNA isolation, pericarp tissue was homogenized in liquid nitrogen to a fine powder. Part of this powder was used for quantification of chlorophylls and carotenoids (see below) and part for RNA extraction as described previously (Lois *et al.*, 2000). Probes were made by labelling cDNA inserts with ³²P-dCTP using the Ready-to-Go kit (Pharmacia). The *DXR* probe was made from the cDNA of clone cLEB8M11 amplified by PCR with Taq DNA polymerase (Gibco BRL) and primers LeDXR5F and LeDXR3R. Probes for *DXS*, *PSY1* and 25S rRNA were made as described by Lois *et al.* (2000). The tomato *ACS2* cDNA used as a probe

contained the last 56 nucleotides of the coding region and most of the 3'-UTR. The 254 bp cDNA was amplified from tomato orange fruit RNA by RT-PCR with Taq DNA polymerase (Gibco BRL) and the primers LeACS2-3F (5'-AAAGTGTGTTTGTACCAC-3') and LeACS2-3R (5'-TTGCATTCGTTAATTAGC-3'). Blotting, hybridization, washes, and exposure were carried out as described previously (Lois *et al.*, 2000).

Antibody production and immunoblot analysis

Antibodies were raised in rabbits against a synthetic peptide of 11 amino acid residues (from position E71 to K81) of the N-terminal region of the deduced *Arabidopsis* DXR protein (Figure 2a). Both the peptide and the antibodies were ordered from Sigma-Genosys (UK) and produced in their facilities. A 1:500 dilution of antiserum from the third bleed of rabbit 2004 was successful in detecting a single band of the predicted size for the mature DXR protein in tomato protein extracts and was therefore used in further experiments. Proteins were extracted from tomato tissues as described by Rodríguez-Concepción *et al.* (1999). For immunoblot analysis, aliquots of 30 µg protein were electrophoresed in 10% SDS-PAGE and electrotransferred to Hybond-P PVDF membranes (Amersham) using the MiniProtean system (BioRad). Blots were blocked in PBS-T (PBS buffer supplemented with 5% w/v powdered non-fat dry milk and 0.1% v/v Tween-20) for 14–18 h at 4°C. Incubation with the antiserum in PBS-T was carried out for 1 h at room temperature. Blots were then washed three times for 10 min in PBS-T and incubated for 1 h at room temperature with an HRP-conjugated secondary antibody (Roche) diluted 1:25 000 in PBS-T. After three washes, cross-reacting bands were detected using the ECL system (Amersham).

In situ hybridization

Tomato orange fruit sections (8 µm) were processed for *in situ* hybridization with digoxigenin-labelled riboprobes as described previously (Lois *et al.*, 2000). Sense and antisense *DXR* riboprobes were obtained from clone Al485694, which contains 364 bp of *DXR* cDNA 3'-end cloned into pBluescript SK⁺. The antisense riboprobe was synthesized by *in vitro* transcription with T7 RNA polymerase after linearization of the plasmid clone with *Nco*I. Similarly, the sense probe was made with T3 RNA polymerase after digestion with *Xho*I. Specific activity of probes was checked by dot blot analyses, and working dilutions of 1:150 in 120 µl hybridization buffer were used. Signal detection was performed colorimetrically with NBT/BCIP (Roche). After 16 h of incubation, reactions were stopped with 10 mM Tris-HCl, 1 mM EDTA (pH 8). Sections were mounted in Aquatex (Merck) for observation.

Chlorophyll and carotenoid measurement

The same pool of frozen powder from pericarp tissue of control and fosmidomycin-treated fruit halves used for RNA extraction was also used for determination of chlorophyll and carotenoid contents as described by Lois *et al.* (2000).

Acknowledgements

We thank D. Frisch (Clemson University Genomics Institute) for the tomato clones, M. Rohmer for the gift of ME, X. Fernández-Busquets and P. Leivar for help with antibody characterization, J.F. Martínez-García and M. Phillips for critical reading of the

manuscript, A. Orozco for valuable technical assistance, and the staff of the Serveis de Camps Experimentals (UB) for the care of plants. This work was supported by grants from the Spanish MEC (DGICT, PB96-0176) and Generalitat de Catalunya (CIRIT, 1999SGR-00032). L.M.L. and S.S.G. received fellowships from the Spanish Ministerio de Educación y Cultura (MEC) and Generalitat de Catalunya, respectively.

References

- Araki, N., Kusumi, K., Masamoto, K., Niwa, Y. and Iba, K. (2000) Temperature-sensitive *Arabidopsis* mutant defective in 1-deoxy-D-xylulose 5-phosphate synthase within the plastid non-mevalonate pathway of isoprenoid biosynthesis. *Physiol. Plant.* **108**, 19–24.
- Bartley, G.E. and Scolnik, P.A. (1993) cDNA cloning, expression during development, and genome mapping of *PSY2*, a second tomato gene encoding phytoene synthase. *J. Biol. Chem.* **268**, 25718–25721.
- Bartley, G.E. and Scolnik, P.A. (1995) Plant carotenoids: pigments for photoprotection, visual attraction, and human health. *Plant Cell*, **7**, 1027–1038.
- Campos, N., Rodríguez-Concepción, M., Seemann, M., Rohmer, M. and Boronat, A. (2001) Identification of *gcpE* as a novel gene of the 2-C-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis in *Escherichia coli*. *FEBS Lett.* **488**, 170–173.
- Cutler, A.J. and Krochko, J.E. Formation and breakdown of ABA. *Trends Plant Sci.* **4**, 472–478.
- Estévez, J.M., Cantero, A., Romero, C., Kawaide, H., Jiménez, L.F., Kuzuyama, T., Seto, H., Kamiya, Y. and Leon, P. (2000) Analysis of the expression of *CLA1*, a gene that encodes the 1-deoxyxylulose 5-phosphate synthase of the 2-C-methyl-D-erythritol-4-phosphate pathway in *Arabidopsis*. *Plant Physiol.* **124**, 95–103.
- Fraser, P.D., Truesdale, M.R., Bird, C.R., Schuch, W. and Bramley, P.M. (1994) Carotenoid biosynthesis during tomato fruit development. Evidence for tissue-specific gene expression. *Plant Physiol.* **105**, 405–413.
- Fraser, P.D., Kiano, J.W., Truesdale, M.R., Schuch, W. and Bramley, P.M. (1999) Phytoene synthase-2 enzyme activity in tomato does not contribute to carotenoid synthesis in ripening fruit. *Plant Mol. Biol.* **40**, 687–698.
- Fray, R.G. and Grierson, D. (1993) Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression. *Plant Mol. Biol.* **22**, 589–602.
- Gillaspy, G., Ben-David, H. and Gruissem, W. (1993) Fruits: a developmental perspective. *Plant Cell*, **5**, 1439–1451.
- Groot, S.P.C. and Karssen, C.M. (1992) Dormancy and germination of abscisic acid-deficient tomato seeds. *Plant Physiol.* **99**, 952–958.
- Harker, M. and Bramley, P.M. (1999) Expression of prokaryotic 1-deoxy-D-xylulose 5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis. *FEBS Lett.* **448**, 115–119.
- Herz, S., Wungsintaweekul, J., Schuhr, C.A., *et al.* (2000) Biosynthesis of terpenoids: YgbB protein converts 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate to 2C-methyl-D-erythritol 2,4-cyclodiphosphate. *Proc. Natl Acad. Sci. USA*, **97**, 2486–2490.
- Julliard, J.H. (1992) Biosynthesis of the pyridoxal ring (vitamin B₆) in higher plant chloroplasts and its relationship with the

- biosynthesis of the thiazole ring (vitamin B₁). *C. R. Acad. Sci. Series III*, **314**, 285–290.
- Julliard, J.H. and Douce, R.** (1991) Biosynthesis of the thiazole moiety of thiamin (vitamin B₁) in higher plant chloroplasts. *Proc. Natl Acad. Sci. USA*, **88**, 2042–2045.
- Khudairi, K.A.** (1972) The ripening of tomatoes. *Am. Sci.* **60**, 696–707.
- Lange, B.M. and Croteau, R.** (1999) Isoprenoid biosynthesis via a mevalonate-independent pathway in plants: cloning and heterologous expression of 1-deoxy-D-xylulose 5-phosphate reductoisomerase from peppermint. *Arch. Biochem. Biophys.* **365**, 170–174.
- Lange, B.M., Wildung, M.R., McCaskill, D. and Croteau, R.** (1998) A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. *Proc. Natl Acad. Sci. USA*, **95**, 2100–2104.
- Lichtenthaler, H.K.** (1999) The 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 47–65.
- Lichtenthaler, H.K., Rohmer, M. and Schwender, J.** (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiol. Plant.* **101**, 643–652.
- Lois, L.M., Campos, N., Rosa Putra, S., Danielsen, K., Rohmer, M. and Boronat, A.** (1998) Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis. *Proc. Natl Acad. Sci. USA*, **95**, 2105–2110.
- Lois, L.M., Rodríguez-Concepción, M., Gallego, F., Campos, N. and Boronat, A.** (2000) Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. *Plant J.* **22**, 503–513.
- Lüttgen, H., Rohdich, F., Herz, S., et al.** (2000) Biosynthesis of terpenoids: YchB protein of *Escherichia coli* phosphorylates the 2-hydroxy group of 4-diphosphocytidyl-2-C-methyl-D-erythritol. *Proc. Natl Acad. Sci. USA*, **97**, 1062–1067.
- Mandel, M.A., Feldmann, K.A., Herrera-Estrella, L., Rocha-Sosa, M. and León, P.** (1996) *CLA1*, a novel gene required for chloroplast development, is highly conserved in evolution. *Plant J.* **9**, 649–658.
- Miller, B., Heuser, T. and Zimmer, W.** (2000) Functional involvement of a deoxy-D-xylulose 5-phosphate reductoisomerase gene harboring locus of *Synechococcus leopoliensis* in isoprenoid biosynthesis. *FEBS Lett.* **481**, 221–226.
- Mueller, C., Schwender, J., Zeidler, J. and Lichtenthaler, H.** (2000) Properties and inhibition of the first two enzymes of the non-mevalonate pathway of isoprenoid biosynthesis. *Biochem. Soc. Trans.* **28**, 792–793.
- Nakatsuka, A., Murachi, S., Okunishi, H., Shiomi, S., Nakano, R., Kubo, Y. and Inaba, A.** (1998) Differential expression and internal feedback regulation of 1-aminocyclopropane-1-carboxylate synthase, 1-aminocyclopropane-1-carboxylate oxidase, and ethylene receptor genes in tomato fruit during development and ripening. *Plant Physiol.* **118**, 1295–1305.
- Rodríguez-Concepción, M., Yalovsky, Y., Zik, M., Fromm, H. and Grisse, W.** (1999) The prenylation status of a novel plant calmodulin directs plasma membrane or nuclear localization of the protein. *EMBO J.* **18**, 1996–2007.
- Rodríguez-Concepción, M., Campos, N., Lois, L.M., Maldonado, C., Hoeffler, J.F., Grosdemange-Billiard, C., Rohmer, M. and Boronat, A.** (2000) Genetic evidence of branching in the isoprenoid pathway for the production of isopentenyl diphosphate and dimethylallyl diphosphate in *Escherichia coli*. *FEBS Lett.* **473**, 328–332.
- Rohdich, F., Wungsintaweeikul, J., Fellermeier, M., Sagner, S., Herz, S., Kis, K., Eisenreich, W., Bacher, A. and Zenk, M.H.** (1999) Cytidine 5'-triphosphate-dependent biosynthesis of isoprenoids: YgbP protein of *Escherichia coli* catalyzes the formation of 4-diphosphocytidyl-2-C-methylerythritol. *Proc. Natl Acad. Sci. USA*, **96**, 11758–11763.
- Rohdich, F., Wungsintaweeikul, J., Lüttgen, H., Fischer, M., Eisenreich, W., Schuhr, C.A., Fellermeier, M., Schramek, N., Zenk, M.H. and Bacher, A.** (2000) Biosynthesis of terpenoids: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase from tomato. *Proc. Natl Acad. Sci. USA*, **97**, 8251–8256.
- Rohmer, M., Knani, M., Simonin, P., Sutter, B. and Sahm, H.** (1993) Isoprenoid biosynthesis in bacteria: a novel pathway for early steps leading to isopentenyl diphosphate. *Biochem. J.* **295**, 517–524.
- Schwender, J., Müller, C., Zeidler, J. and Lichtenthaler, H.K.** (1999) Cloning and heterologous expression of a cDNA encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase of *Arabidopsis thaliana*. *FEBS Lett.* **455**, 140–144.
- Sharkey, T.D., Chen, X. and Yeh, S.** (2001) Isoprene increases thermotolerance of fosmidomycin-fed leaves. *Plant Physiol.* **125**, 2001–2006.
- Takahashi, S., Kuzuyama, T., Watanabe, H. and Seto, H.** (1998) A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis. *Proc. Natl Acad. Sci. USA*, **95**, 9879–9884.
- Walter, M.H., Fester, T. and Strack, D.** (2000) Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the 'yellow pigment' and other apocarotenoids. *Plant J.* **21**, 571–578.
- Zeevaert, J.A.D. and Creelman, R.A.** (1988) Metabolism and physiology of abscisic acid. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **39**, 439–473.
- Zeidler, J., Schwender, J., Müller, C., Wiesner, J., Weidemeyer, C., Beck, E., Jomaa, J. and Lichtenthaler, H.K.** (1998) Inhibition of the non-mevalonate 1-deoxy-D-xylulose 5-phosphate pathway of plant isoprenoid biosynthesis by fosmidomycin. *Z. Naturforsch.* **53c**, 980–986.

Accession number AF331705(tomato *DXR* cDNA)